Faculty of Resource Science and Technology

Seed Transformation of *Morinda citrifolia* via *Agrobacterium tumefaciens*

Fazliza binti Mohd. Ali

Supervisor: Dr. Hairul Azman @ Amir Hamzah bin Roslan

Co-supervisor: Ms. Safarina binti Ahmad

Bachelor of Science with Honours

(Resource Biotechnology)

2008

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This project is submitted in partial fulfillment of the requirements of the degree of

Bachelor of Science with Honours

(Resource Biotechnology)

Department of Molecular Biology

Faculty of Resource Science and Technology

UNIVERSITI MALAYSIA SARAWAK

2008

ACKNOWLEDGEMENT

First of all, thank God for blessing me with the strength and will to complete this project on time. Sincere thanks are extended to my supervisor, Dr. Hairul Azman bin Roslan, for his generosity of time, expertise and attention in guiding me throughout the completion of this project. I am also indebted to Ms. Safarina binti Ahmad, my co-supervisor, for her kind guidance and insightful comments especially in writing up of this dissertation. Heartfelt thanks are also for my family, labmates and fellow coursemates for their help and encouragement that make this project possible. For everyone who has contributed directly or indirectly in the completion of this project, all your kindness and patience are highly appreciated.

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Resource Biotechnology
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Morinda citrifolia, known commercially as noni, is considered as a medicinal plant with various therapeutic values. The significant values of this plant lead an attempt to produce a better plant by introducing foreign genes to the plant through transformation. Prompted by successes in earlier studies of *Agrobacterium*-mediated *in planta* transformation procedures in *Arabidopsis*, similar approach is applied to transform *M. citrifolia*. This study aims to carry out *Agrobacterium*-mediated *in planta* transformation on *M. citrifolia* by using seed transformation method and also to reconstruct transformation vector carrying a novel promoter. The presence of the GUS fragment that has been detected by PCR using GUS-e primers suggests that this plant has been successfully transformed. However, an attempt for reconstructing vector with the novel promoter was unsuccessful.

Keywords: *Morinda citrifolia*, *in planta* transformation, seed transformation.

ABSTRAK

Morinda citrifolia yang lebih dikenali sebagai noni merupakan sejenis tumbuhan yang kaya dengan khasiat dari segi perubatan. Nilai-nilai perubatan yang terdapat pada tumbuhan ini telah mendorong pelbagai usaha untuk meningkatkan mutunya melalui kaedah transformasi. Kejayaann kajian-kajian lepas mengenai transformasi in planta melalui Agrobacterium pada Arabidopsis telah mendorong untuk mengadaptasikan kaedah yang sama untuk transformasi ke atas M. citrifolia. Matlamat bagi kajian ini adalah untuk melakukan transformasi in planta berperantarakan Agrobacterium pada *M. citrifolia melalui kaedah transfomasi biji benih dan juga untuk membina vektor transformasi yang membawa promoter baru. Kehadiran fragmen GUS yang telah diperolehi daripada PCR mennggunakan set primer GUS-e mencadangkan bahawa tumbuhan ini telah berjaya ditransformasi. Walaubagaimanapun, usaha untuk membina vektor baru adalah tidak berjaya.*

Kata kunci: Morinda citrifolia, transformasi in planta, transfomasi biji benih.

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LIST OF ABBREVIATIONS

AGE	agarose gel electrophoresis
BAR	native phosphinothricin acetyltransferase
bp	base pair
CIA	chloroform-isoamyl alcohol
CTAB	cetyltrimethyl ammonium bromide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetra acetate
GUS	β-Glucuronidase
kb	kilo base pair
ml	milliliter
mM	milimolar
PCR	Polymerase Chain Reaction
TAE	Tris-Acetate-EDTA
V	volts
μg	microgram
μl	microliter
μM	micro molar

CHAPTER 1

INTRODUCTION

Morinda citrifolia, known commercially as noni, of the family Rubiaceae is considered as a medicinal plant with various therapeutic values of every part of the plant. In the Tropics, this plant seems to have been much valued medicinally and it is normally cultivated for its roots, leaves and fruits (Zin *et al.,* 2002). This plant is also one of the most significant sources of traditional medicines among Pacific island society and it has been used by Polynesians since more than 2000 years ago for its wide range of remedial effects, including antimicrobial, antitumor, analgesic, anti-inflammatory and also for immune enhancing effects (Nelson, 2006a). Claims of these health benefits of noni through medicinal history have been revealed and proved by scientific studies (Wang *et al.,* 2002). Subsequently, *M. citrifolia* has become an economically important plant worldwide, in recent years, through a variety of health and cosmetic products made, mainly, from its leaves and fruits (Nelson, 2006a).

The significant values of this plant as an important crop to the society has lead an attempt to produce a better plant by introducing foreign genes to the plant through transformation. Previous studies of *in planta* transformation procedures, especially the floral transformation, in *Arabidopsis* (Clough and Bent, 1998; Bent, 2000; Desfeux *et al*., 2000) have been a tremendous success and it is inspiring to adapt this method for the transformation of other plant species such as *M. citrifolia*. Previously, the concurrent effort to improve this crop is mainly involving the selection of varieties with desirable traits (McClatchey, 2002). This plant is chosen to be transformed in this study for the

reason that there is no research on the transformation of *M. citrifolia* that involving manipulation at the molecular level has been published so far. Thus, this study is carried out in order to determine the possibility of *M. citrifolia* to be transformed by the mean of *in planta* transformation via *Agrobacterium tumefaciens*.

Bent (2000) has reported that the *in planta* Agrotransformation is a fast and effective technique to transform plant cells without the step of tissue culture. An attempt to avoid *in vitro* culture and regeneration of the whole plant from the transformed single cell is due to their requirements for highly-skilled worker and the time-consuming effort (Grabowska & Filipecki, 2004). Furthermore, mutations that range from single base changes or small rearrangements to the loss of the entire chromosomes are often produced during the plant regeneration from single transformed cells. So, methods such as seed transformation by Feldmen & Marks (1987) (as cited in Grabowska & Filipecki, 2004), vacuum-infiltration by Bechtold *et al.* (1993), floral dip by Clough & Bent (1998), and floral spray transformation by Chung *et al.* (2000) have been developed in which *in vitro* techniques can be eliminated and therefore reducing the risk of somaclonal variation that is often found in the regenerated plants (Rakoczy-Trojanowska, 2000 as cited in Grabowska & Filipecki, 2004).

In this study, one of the *in planta* transformation methods, which is seed transformation, was performed on *M. citrifolia*. In this method, the mature seeds of the plant were incubated in the inoculation medium for the transformation process to take place. But beforehand, the *A. tumefaciens* that will be used as the bacterial suspension in the inoculation medium will be transformed to carry plasmids that allow for the selection of putative transformants.

The plasmid that has been utilized for transformation in this study was pGSA1131 with *gus* and *bar* genes for selection. The presence of the GUS and BAR fragments was confirmed by PCR in which primer sets of GUS-e F/GUS-e R and BAR-3 F/BAR-3 R were used, respectively. This plasmid was then transformed into *A. tumefaciens* LBA4404 for the plant transformation.

The success of the transformation was determined by analyzing the non-germinated transformed seeds and also leaves of seedlings germinated from transformed seeds. DNA from these plant tissues was extracted and Polymerase Chain Reaction (PCR) was performed for confirmation to detect the presence of inserts in the plant tissues.

The objectives of this study are listed here:

- To transform *M. citrifolia* via *A. tumefaciens* using seed transformation method
- To re-construct plasmid that carry novel promoter, together with *gus* gene, as a putative transformation vector

CHAPTER 2

LITERATURE REVIEW

2.1 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a pathogenic Gram-negative soil bacterium that has the capacity to genetically engineer plants in nature by transferring the T-DNA region of its Ti plasmid to a recipient plant cell (Hooykaas & Schilperoort, 1985; Steck, 1996). The plasmid is called Ti plasmid due to its tumor-inducing property in the natural gene transfer system which causes tumors in many plants (Chrispeels & Sadava, 2003). When the bacteria infect a wound site on the plant, a part of its large Ti plasmid that is called the transfer DNA (T-DNA), will be cut out by enzymes, coated by proteins and transferred into a plant cell so that it will become randomly integrated into the plant genome (Smith & Wood, 1991; Chrispeels & Sadava, 2003). As a result of the expression of the transferred genes from the bacteria, tumors called crown galls develop at the infection sites (Hooykaas & Schilperoort, 1985). The tumorous growth of undifferentiated cells will produce specialized compounds, called opines, which can be used as a sole source of carbon, nitrogen and energy by the bacteria and thus resulting in their growth and proliferation (Brown *et al.*, 1987; Smith & Wood, 1991).

Agrobacterium is capable to carry out a true genetic manipulation in plants because it contains all the functions necessary for the transfer, stable incorporation and expression of the transferred genetic information in the T-DNA (Brown *et al*., 1987). After the mechanism of DNA transmission was understood, it is clear that the Ti system could be applied as a vector in introducing genetically engineered DNA into plants

(Madigan & Martinko, 2006). Plasmids have been constructed that they are no longer has genes that cause disease, yet retain genes responsible for transfer of the T-DNA into the plant cells (Brown *et al*., 1987; Madigan & Martinko, 2006). These plasmids are called 'disarmed' plasmids and they are manipulated widely for the production of transgenic plants. Plant cells will be transformed when the bacteria transfer their T-DNA region together with the foreign genes that will be expressed in plant cells (Brown *et al*., 1987; Smith & Wood, 1991).

2.2 *In planta* transformation via *Agrobacterium tumefaciens*

Agrobacterium-mediated transformation is one of the most common methods for introduction of foreign DNA into plant cells (Bent, 2000). Transformation of isolated plant cells such as protoplasts and callus culture cells is usually straightforward and more feasible (Gelvin & Schilperoort, 1998 as cited in Bent, 2000). However, the requirements of particular preparation of cells and tissues, DNA introduction, selection of transformed cells and plant regeneration have become the great obstacles and lots of time, skilled worker and relatively expensive facilities are needed (Grabowska & Filipecki, 2004).

In planta transformation methods, such as seed transformation and floral transformation, have enabled the transformation of plants without the requirement for regeneration protocols (Tague, 2000). With these methods of transformation, most transformed progeny are genetically uniform and the somaclonal variation associated with tissue culture and regeneration is minimized (Clough & Bent, 1998). Moreover, the simplicity and high effectiveness of *in planta* transformation have provide a promising tool for further investigation of T-DNA integration process into the plant genome

(Radchuk *et al.*, 2005). Radchuk *et al.* (2005) also reported that studies have revealed that multi-gene transfer is possible by *in planta* transformation.

Agrobacterium-mediated *in planta* transformation is a non-tissue culture approach that has been used earlier to transform cells in or around the apical meristems, and the protocol has undergone several modifications since 1990s that lead to the improvement in plant transformation (Bent, 2000). In 1993, Bechtold and co-workers reported success in transformation by 'vacuum infiltration' that has become a crucial stage of the revolution in *Arabidopsis* transformation (Bent, 2000). As the name implies, the infiltration of *Arabidopsis* with *Agrobacterium* solution was achieved with the aid of vacuum pressurizing device. This relatively simple method was not time-consuming and did not require sophisticated equipments or skilled labor, yet resulted in much higher transformation efficiency (Bechtold *et al*., 1993 as cited in Grabowska & Filipecki, 2004). This method also has overcome the main difficulties in generating the exceptionally large numbers of insertion mutations of the *Arabidopsis* genome (Galbiati *et al*., 2000). Grabowska & Filipecki (2004) reported successful in transformations of different plant species, such as alfalfa, radish, pakchoi and petunia, via infiltration method. Nevertheless, vacuum infiltration is not an easy method to be applied to other plants which are too large for the vacuum step (Chung *et al*., 2000).

Therefore, simpler methods for the floral transformation have been developed in which the *Agrobacterium* solution can be directly applied to the young developing flowers without using the vacuum infiltration. Clough & Bent (1998) came up with floral dip method as a simplified technique for *Agrobacterium*-mediated transformation in which the vacuum infiltration process was eliminated. They simply dipped the

developing floral tissues of *Arabidopsis* into a solution containing *A. tumefaciens*. The inclusion of Silwet L-77 in the inoculation medium allowed the elimination of vacuum step since the surfactant reduces the surface tension and keeps the bacterial suspension longer on the plant for better penetration of bacteria into the cellular spaces (Clough & Bent, 1998). Since the primary targets for productive transformation in the floral transformation are ovules, the growth stage of plants during inoculation is critical in which the locule should remain open and the developing ovules are accessible for transformation by *Agrobacterium* (Desfeux *et al*., 2000).

In 2000, floral spray and floral dip methods were used by Chung and co-workers to replace the vacuum step in the *Agrobacterium*-mediated transformation in *Arabidopsis*. They claimed that the floral spray method as an alternative way for transforming foreign genes in *Arabidopsis* and it may be applicable to other plants of interest. Grabowska & Filipecki (2004) also claimed that the simplification of the infiltration method by vacuum elimination made the transformation of other plants possible. Recently, successful *in planta* transformations via *A. tumefaciens* of kenaf plants (*Hibiscus cannabinus*) and wheat (*Triticum aestivum*) has been reported by Kojima *et al*. (2004) and Supartana *et al*. (2006), respectively.

2.3 *Morinda citrifolia*

Morinda citrifolia, also known as noni or ‘mengkudu’, is one of the important medicinal plants of ancient Pacific Island cultures that have become well known throughout the world for its wide range of values and uses, especially in medical and therapeutic fields. *M. citrifolia* is native to Southeast Asia and Australia, and now this small, evergreen

shrub has a pantropical distribution (Nelson, 2006a). This plant is frequently identified as a part of primary forests or shrub vegetation and is widely distributed due to its ability to self-pollinate as part of their adaptation for colonizing new terrain (McClatchey, 2002). This evergreen shrub produces fruits with characteristics of a strong butyric acid smell and flavor once they ripe although there is much variation in overall plant form, including the size of fruits and also the size and morphology of leaves (Nelson, 2006a; Chan-Blanco *et al.*, 2005).

Nelson (2006a) reported that all parts of *M. citrifolia* have traditional and/or modern uses. Generally, parts of this plant such as stem, root, bark, leaf and fruit have been used traditionally to treat many diseases including diabetes, hypertension and venereal diseases. This plant was also originally cultivated as a dye plant since the bark contains a red pigment while a yellow pigment can be obtained from the roots (Nelson, 2006b). Over the past twenty years, great interest in noni has developed since its beneficial properties and uses have been widely-known (McClatchey, 2002). Chan-Blanco *et al*. (2005) reported that the noni fruit has antibiotic and antioxidant properties, while other properties such as antibacterial, alterative, antiparasitic, stomachic, anticancer, anti-inflammatory, antiarthritic, analgesic and antihypertensive of the plant has been reported by Tierra, as cited in Fahs (2002). Most natural antioxidants that can be found normally as phenolic or polyphenolic compounds can neutralize free radicals, which serve an important role in the prevention of carcinogenity, cardiovascular, and also neurodegenerative changes associated with aging (Zin *et al.*, 2002; 2006). Besides that, Zin and co-workers (2006) also reported that the presence of flavonoids and

anthraquinones in various parts of the plant have contributed to the wide range of the biological effects that it serves.

People around the world consume noni for treating various ailments in many different ways according to their cultures (Fahs, 2002). For an instance, the flowers are employed by Samoan in treating styes, while people in Tonga used vapour from the broken leaves for the same purpose. In Hawaii, fetid oil that was extracted from the syncarp of noni is used as insect repellant and also used on hairs to treat head lice (Nelson, 2006b). Other common uses of noni include a tonic prepared from the fruit is taken for treating diabetes, high blood pressure, tuberculosis and loss of appetite (Fahs, 2002; Nelson, 2006b). Besides that, Tierra (as cited in Fahs, 2002) also has documented noni's uses that include the treatment of chronic respiratory conditions and many digestive disorders such as diarrhea, constipation, indigestion and internal parasites as well. A scientific basis for the health benefits, as claimed in traditional medicinal practice, for *M. citrifolia* is proved by studies which stated that a high consumption of fruits and vegetables containing phenolic antioxidants can slow the process of atherosclerosis and also reduce the risk of cancer and many other diseases (Zin *et al.*, 2006).

Many studies have been carried out mostly to determine the nutritional and therapeutic properties of the *M. citrifolia* (Tierra, as cited in Fahs, 2002; Zin *et al.*, 2002; Chan-Blanco *et al.*, 2005; Zin *et al.*, 2006) while other researches have been done to study the diversity of the species and its close relatives (McClatchey, 2002; Mortan, as cited in McClatchey, 2002; Nelson, 2006a). There is no published research on the transformation of *M. citrifolia* available so far.

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant materials preparation

Soft, fully ripe *M. citrifolia* fruits were suspended in water in order to separate the seeds from the flesh easily. Then, the fruit was mashed and the seeds were collected. For long storage, the flesh was completely removed from the seeds, followed by rinsing with tap water and air-drying, as recommended by Nelson (2006a). The seeds were then kept in a polypropylene tube and stored at 4^0C. Twenty five seeds were randomly selected to be planted on soil as controls. The seeds were vernalized in tap water at 4^0C for two days before they were planted in moistened potting soil. The planted seeds were allowed to germinate and grow under natural conditions.

3.2 Culture of *A. tumafaciens* and *E. coli*

E. coli XL1Blue (Stratagene) were used for cloning purposes and *A. tumefaciens* LBA4404 (gift from Caddick, The University of Liverpool) were used to transform *M. citrifolia*. All bacterial strains were maintained in LB media (Luria Bertani Broth, Miller), except when stated otherwise. *A. tumefaciens* LBA4404 were grown at 28^0C with 150 rpm shaking, while the *E. coli* XL1Blue cultures were grown at 37^0C with 150 rpm shaking.

3.3 Competent cells preparation and bacterial transformation

3.3.1 Competent cells preparation and transformation of *E. coli*

Competent cells for *E. coli* XL1Blue were prepared according to calcium chloride method by Sambrook *et al.* (1989) with some modifications. A 10 ml of LB liquid medium was inoculated with a single colony of *E. coli* XL1Blue and the culture was grown overnight at 37^{o}C with shaking at 150 rpm. One milliliter of the culture was added to a 50 ml polypropylene centrifuge tube containing 10 ml of pre-warmed LB media without antibiotics. The culture was allowed to grow until the OD_{600} reached approximately 0.45 to 0.5. After that, the cells were transferred into an ice-cold 50 ml polypropylene centrifuge tube and were then kept on ice for 10 minutes before centrifuged at 3500 rpm at 4^{o}C for 5 minutes by using KUBOTA 8800 Centrifuge. The supernatant was discarded and the cells were washed by gently resuspending them in 5 ml iced-cold 100 mM $CaCl_2$. The cells were then again kept on ice for 10 minutes and followed by centrifugation at 3500 rpm at 4^{o}C for 5 minutes. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 1.6 ml of cold, sterile 100 mM $CaCl_2$ before it was incubated on ice for an hour. After that, 20% (v/v) pure glycerol to cell suspension was prepared by adding and mixing 320 µl of pure glycerol to the cell suspension. Then, 200 µl aliquots were transferred into 1.5 ml microcentrifuge tubes before being stored at -80^{o}C.

Prior to begin the transformation, an incubator shaker and water bath were pre-heated to 37^{o}C and 42^{o}C, respectively. Then, 1 µl of plasmid that has been equilibrated to room temperature was added to the bottom of the 1.5 ml microcentrifuge tube that has been pre-cooled on ice and labeled. *E. coli* XL1Blue competent cells were removed from

the freezer and placed in a 50% ice/distilled water bath for exactly 5 minutes, followed by gentle flicking to mix the competent cells. Next, 50 µl competent cells were added to the 1.5 ml microcentrifuge tube on ice and the tube was flicked gently before it was left on ice for 20 minutes. Then, the cells were heat-shocked in a heating block (Thermo Bath ALB64) for exactly 45 seconds at 42^{o}C and then returned to ice for exactly two minutes. Subsequently, 950 µl LB media was added to the transformation and mixed by flicking gently before it was put in incubator-shaker at 37^{o}C for 90 minutes. After 60 minutes, LA (LB Agar, Miller) plate with appropriate antibiotic was placed in laminar flow hood with lids off to dry out for 30 minutes. Once dried, 50 µl of the transformation culture was pipetted onto the plate and spread by using a sterile bent glass rod. Next, the plate was sealed with parafilm and placed in 37^{o}C incubator for overnight.

For selection of *E. coli* that harbored pGSA1131, LA plates containing 35 µg/ml chloramphenicol were used, while LA plates containing 50 µg ml^{-1} ampicillin were used to select *E. coli* that harbored pAGS or pGE1.

3.3.2 Competent cells preparation and transformation of *A. tumefaciens*

For competent cells preparation and transformation of *A. tumefaciens* LBA4404, a freeze-thaw method as described by Roslan (1999) was utilized. A 50 µl of *A. tumefaciens* LBA4404 stock culture was added to 100 ml of LB liquid medium containing 100 µg/µl rifampicin, and the culture was grown at 28^{o}C with 150 rpm shaking for two days. The culture was transferred into two 50 ml polypropylene centrifuge tubes and allowed to cool on ice for 30 minutes, followed by centrifugation at 5000 rpm for 5 minutes at 4^{o}C by using KUBOTA 8800 Centrifuge. Then, the supernatant was discarded and the pellet

was resuspended in 1 ml of cold 20 mM $CaCl_2$ solution. The cell suspension was transferred into 1.5 ml microcentrifuge tubes in 100 µl aliquots and the tubes were then snap-frozen in liquid nitrogen before stored at -80^{o}C.

Before being used for transformation, the frozen competent *A. tumafaciens* LBA4404 cells were thawed on ice. One microliter of plasmid was added to the cells and the tube was frozen into liquid nitrogen. Then, the tube was incubated in a 37^{o}C water bath for 5 minutes. Next, the cells were allowed to recover by transferring the cells to 1 ml of LB liquid media and followed by incubation at 28^{o}C with 150 rpm shaking for three hours. After that, 50 µl of the transformation culture was spread on LA plates containing appropriate antibiotic, and the plates were then incubated at 28^{o}C for two days.

3.4 Isolation of double-stranded plasmid DNA from bacterial culture

Mini-prep isolation of double-stranded plasmid DNA from the putative transformed bacterial culture was carried out in order to do PCR and also for restriction analysis. A single colony that grew on the LA+chloramphenicol plate was inoculated into 10 ml LB liquid medium and grown for overnight at 28^{o}C and 37^{o}C, for *A. tumefaciens* and *E. coli* respectively, with shaking at 150 rpm. Then, bacterial cells from the overnight culture were harvested by transferring the culture equally into two 50 ml polypropylene centrifuge tubes and followed by centrifugation at 8000 rpm for 2 minutes at room temperature by using KUBOTA 8800 Centrifuge. The supernatant from both tubes were then removed carefully and the pellets were recentrifuged for 1 minute. After that, any traces of liquid media were completely removed from the tubes and the cell pellets were

resuspended in 100 µl of Solution I (see Appendix A), followed by vortexing for 10 seconds. The suspensions were transferred individually into two 1.5 ml microcentrifuge tubes and afterwards the tubes were always kept on ice. Then, 100 µl of Solution II (see Appendix A) was added to each cell suspension and was gently mixed by inverting the tubes for ten times. The tubes were then left at room temperature for 5 minutes to allow the lysis reaction to occur. After that, 300 µl of Solution III (see Appendix A) was added and the tubes were inverted ten times to mix. Then, the precipitates obtained were pelleted by centrifuging at 10000 rpm for 5 minutes at room temperature by using Hettich Zentrifugen EBA21 and supernatants were carefully transferred into two sterile 1.5 ml microcentrifuge tubes. Next, the DNA was precipitated by adding 2 volume of cold absolute ethanol and the contents were gently mixed by inverting the tubes for at least ten times. The DNA was then pelleted by centrifuging at 13000 rpm for 5 minutes at room temperature and the supernatants were discarded. The pellets were washed with 500 µl of 70% ethanol and recentrifuged at 13000 rpm for 2 minutes. After that, the supernatants were discarded as much as possible and the DNA pellets were allowed to air dry for 15 minutes at room temperature in the laminar flow hood. The DNA pellets were then resuspended in 50 µl of sterile distilled water and subsequently stored in -20^{o}C.

For plasmid that has been isolated by using GF-1 Plasmid DNA Extraction Kit from Vivantis, all procedures were carried out according to supplier's recommendation.